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Improving Growth and Salinity Tolerance of Carob Seedlings (*Ceratonia siliqua* L.) by *Azospirillum* Inoculation

[1,2]El-Refaey F.A. El-Dengawy, [2]Ahmed A. Hussein and [2]Saad A. Alamri

[1]Department of Pomology, Faculty of Agriculture,
Damietta Branch, Mansoura University, Damietta, Egypt
[2]Department of Biology, Faculty of Science, King Khalid University, Abha, Saudi Arabia

Abstract: Carob has been neglected with respect to both cultural practices and research and development. The current study was carried out for two successive seasons to find a practical method for extension of carob cultivation under saline water irrigation and to evaluate the efficacy of the *Azospirillum* inoculation in the development of salinity tolerance of the carob seedlings under salinity stress (4.69EC, 9.38EC and 14.07EC of seawater). The results showed that the seedling growth characteristics particularly seedling dry weight, the new leaf area and root characters showed significant progressive reductions with increasing salinity levels. But these reductions were significantly ameliorated by *Azospirillum* inoculation. Regarding the leaf mineral and proline content, the saline irrigated seedlings either inoculated or non-inoculated with *Azospirillum* contained significantly higher Na^+, Cl^- and proline concentrations and significantly lower K^+ and N concentrations in leaf than that of the normal irrigated control. However, applying *Azospirillum* inoculum in combination with saline water significantly decreased Na+ and Cl- and increased K+, N and proline concentrations in the seedling leaves under all salinity levels. Also, K/Na ratio was significantly increased by *Azospirillum* inoculum under the most tested levels of salinity. Applying *Azospirillum* inoculum gave significantly higher total phenols concentration than uninoculated seedlings. The SCC percentage was significantly higher in saline water irrigated seedlings at 14.07EC with or without Azospirillum inoculum than the other tested treatments. Chlorophyll concentration in leaves of the treated seedling was significantly decreased with rising levels of salinity. Applying *Azospirillum* inoculum increased chlorophyll concentration in seedling leaves under all salinity levels compared to the uninoculated ones. Moreover, applying *Azospirillum* inoculum in combination with saline water significantly increased survival ratio of the seedlings and decreased injured leaves (%) compared to the uninoculated ones under the same level of salinity. It can be conclude that application of *Azospirillum* inoculum can initiate systemic resistance of carob seedling to salt stress and improve growth.

Key words: Carob seedlings · Salinity stress · *Azospirillum* · Seedling characteristics · Physiological parameters

INTRODUCTION

The carob *(Ceratonia siliqua* L.*)* is found in a range of the wild habitat of many Mediterranean countries including Cyprus, Egypt, Italy, Lebanon, Libya, southern Jordan, Syria, Tunisia and Turkey, representing different genetic resources [1]. The origin of carob is not clear as it has undergone extensive cultivation since ancient times [2]. Carob is also widely planted as an ornamental and shade tree in some countries as USA and Australia [3]. Besides, all parts of the carob tree, e.g. pods, seeds and woods have great economic aspects [4].

Azospirillum was discovered as a growth promoter of cereals. Because much *Azospirillum* research is still conducted on cereal, the perception of *Azospirillum* as a plant-growth-promoting bacterium (PGPB) for cereals is still widespread. This ignores the multitude of studies in which *Azospirillum* affected and promoted the growth of numerous other species, including trees, cacti, vegetables, fruit, flowers, medicinal plants and spices [5]. This review proposed that *Azospirillum* be considered a non-specific PGPB. Successful applications and research in these areas are progressing slowly and most studies are conducted under in vitro conditions. Research in these areas is

Corresponding Author: El-Refaey F.A. El-Dengawy, Department of Pomology, Faculty of Agriculture, Damietta Branch, Mansoura University, Damietta, Egypt.

definitely needed, as *Azospirillum* is tested under stressed environmental conditions.

Being a legume tree, carob like most Caesalpinioideae does not nodulate and thus is unable to fix nitrogen [6]. Therefore, the application of bioinoculant, plant-growth promoting rhizobacteria such as *Azospirillum*, may improve the performance of carob plants by nitrogen fixation by a mechanism other than nodulation.

Numerous cultivated soils worldwide are becoming more saline mainly from the use of marginal irrigation water, from excess fertilization and various desertification processes. Salinity is the major environmental factor limiting plant growth and productivity [7]. Inoculation with *Azospirillum sp.* under saline stress conditions is therefore commonplace. Prior findings showed that common agricultural *Azospirillum* strains tolerated high salinity (≤2%). Improved mineral uptake by plants was suggested as a major contribution of *Azospirillum* inoculation [8]. The effects of salt on *Azospirillum*-plant interactions is a major factor to be considered in applied studies.

The inoculation of plants with *Azosprillium* has also been reported to improve various growth parameters including plant biomass, nutrient uptake, tissue N-content, plant height, leaf area and root: shoot ratio [9], total dry mater and seed yield in Indian mustard [10]. Since salt stress involves both osmotic and ionic stress [11, 12], growth suppression is directly related to total concentration of soluble salts or osmotic potential of soil water [13, 14]. Suppression of growth occurs in all plants, but their tolerance levels and rates of growth reduction at lethal concentrations of salt vary widely among different plant species. Salt stress affects all the major processes such as growth, photosynthesis, protein synthesis and energy and lipid metabolism [15, 16]. Some researches showed that proline accumulation in plants may be a symptom of stress in less salinity-tolerance species and suggested that it plays multiple roles in plant stress tolerance. Proline may act as a mediator of osmotic adjustment [17] protects macromolecules during dehydration [18].

Although the beneficial effects of *Azospirillum brasilense* was previously reported on several plants, no attention was paid to *Azospirillum*-legume dipartite symbioses under salinity stress. Also, in spite of the current expansion in cultivated area in Egypt and Saudi Arabia which accounts for several millions of acres, the prevailing drought has magnified the threat by the associated salinity stress to agriculture and forestry. Therefore, the use of seawater and saline groundwater irrigation might help the arid zone cultivation systems to meet the increasing demand for both food and feed.

Carob tree is known to survive extreme adverse environmental conditions including salinity [4]. On the other hand, seedling establishment is very slow and significantly retarded under salinity conditions [19, 20]. The aim of the current research was to find a practical method for extension carob cultivation under saline water irrigation and to evaluate the efficacy of the *Azospirillum* inoculation in the development of salinity tolerance of the carob seedlings under salinity stress.

MATERIALS AND METHODS

Plant Materials and Experimental Procedure: This experiment was conducted during the two seasons of 2007/2008 and 2008/2009 in a greenhouse and laboratories at the Faculty of Science, King Khalid University, Abha, Saudi Arabia. Carob seeds cv. "Sfax" (*Ceratonia siliqua* L.) were collected from freshly harvested repining pods brought from Tunisia. Seeds were mechanically scarified and soaked in water at 30°C for 48 h to stimulate germination. The seeds were sown in perforated black polyethylene bags (10 seeds for each) filled with 3Kg of sand and clay (2:1, v/v, respectively). The bags were watered regularly under greenhouse. The temperature in greenhouse was 28-32°C. Five months after sowing, the seedlings of uniform vigor were selected and transplanted singly into perforated 20 cm pots filled with the prescribed medium. One month after the transplantation, the healthy seedlings were classified into 8 similar groups (10 seedlings for each). Each group was arranged into 5 replicates (2 seedlings for each) and subjected to one of the following treatments: irrigation with tab water only (control, T1); *Azospirillum* inoculation and tab water (T2); 4.69EC seawater irrigation (T3); *Azospirillum* inoculation and 4.69EC seawater irrigation (T4); 9.38EC seawater irrigation (T5); *Azospirillum* inoculation and 9.38EC seawater irrigation (T6); 14.07EC seawater irrigation (T7); *Azospirillum* inoculation and 14.07EC seawater irrigation (T8). The irrigation treatments were supplied twice a week (250 ml/pot). The mentioned levels of salinity were prepared by dilution of seawater stock with tab water and added at the mentioned scheduled irrigation intervals from start to end of experiment.

Azospirillum Inoculum and Inoculation Technique: Azospirillum lipoferum strain was obtained from microbiological department, Damitta Faculty of Agriculture, Mansoura University, Egypt. Semi-solid malate medium [21] was inoculated with *Azospirillum lipoferum* and incubated at 30°C for 7 days as an inoculum. The number of viable cells was 10^8 CFU/ml. For inoculation of *Azospirillum*, soil around the roots was carefully moved aside without damaging the roots. The inoculum suspension of 20 ml/pot was poured around the roots and soil replaced. In the control treatments, water was added in the same equal volume of inoculum suspension.

Growth Characteristics of Seedlings: Ten seedlings of 9-months-old were collected from each treatment (2 seedlings/replicate) and shoot length (cm), stem diameter (mm) at 5cm above the soil surface, new leaves area (NLA, cm^2), dry weight (DW) of both seedling and root (g), root length (cm) and root branch number were measured.

Leaf Mineral Concentration Determination: Nitrogen concentration was extracted and determination by micro-kjeldahl method as described by Sadasivam *et al.* [22]. Na and K were extracted according to [23]. Leaf fine dry powder (0.2 g) was microwave digested with nitric/hydrogen peroxide, filtered through qualitative filter paper and used for Na^+ and K^+ determinations by flame photometry [24]. The chloride concentration was quantitatively extracted with water and determined according to [25].

Biochemical Determination

Proline Determination: Proline concentration was determined following the method of [26]. Leaf samples were harvested at the end of the experiment. A 0.2 g of fresh weight was mixed with 5.0 ml aliquot of 3% (W/V) sulfosalicylic acid in glass tubes covered at the top and boiled in a water bath at 100°C. The mixture was centrifuged at 2000 g for 5min at 25°C. A 200 µl aliquot of the extract was mixed with 400 µl distilled water and 15 ml of the reagent mixture (30ml glacial acetic acid, 20ml distilled water and 0.5 g of ninhydrin) and boiled at 100°C for 1h. After cooling the mixture, we added 6.0ml of toluene. The chromophore containing toluene was separated and absorption at 520 nm was read, using toluene as a blank. Proline concentration was calculated using L-proline for the standard curve.

Soluble Carbohydrates Concentration (SCC) Determination: The SCC was extracted according to [27]. Weight of 0.1g of leaf fine dry powder was boiled in 10 ml distilled deionized water under shaking for 45 min and then filtered through qualitative filter paper. An aliquot of this filtrate was used for SCC determination according to [28] using D (+)-glucose as standard.

Chlorophyll Determination: Four leaf discs (0.25 cm^2 each) were sampled from the leaves avoiding major veins. Chlorophyll was eluted from the discs by submerging them in 2 ml of N,N-dimethylformamide in the dark for at least 72 h. The amount of absorbance was read at 647 nm and 664 nm with UV-vis spectrophotometer (Model UV1601PC, Shimadzu) and used to calculate leaf total chlorophyll concentrations according to equations of [29].

Total Phenols Concentration: The phenolic concentrations were extracted as described by [30] with modifications. Weight of 0.2g of leaf fine dry powder was homogenized in 80% ethanol. The homogenate was boiled for 15 min, centrifuged 10,000rpm for 10 min and the supernatant saved. The pellet was re-extracted as above and the two supernatants were pooled and evaporated to dryness. The residue was dissolved and completed to a known volume with distilled water and used for phenols measurement using Folin-Ciocalteau reagent [31] with catechol as standard.

Leaf Diffusion Resistance for Water Vapor (LRWV) Measurement: The measurements of LRWV were determined from readings of a Delta-T, AP4 porometer, 128 Low Road Burwell, Cambridge CB5 0EJ, UK, on the median portion of the youngest fully expanded leaf avoiding the mid rib.

Tolerance Index (Ti): For inoculated and non-inoculated carob seedlings, the Ti to salinity levels was determined according to [32] with some modification as: Ti = (Seedling D.W. at any salinity level/seedling D.W. at 0.0 level of salinity) X 100.

Azospirillum Efficacy: At each salinity level, the *Azospirillum* efficacy (Azo.E) on the biomass of seedling was calculated according the following equation [33]:

Azo.E. = (D.W. of inoculated seedling at a specified level of salinity / D.W. of non-inoculated seedling at the same level of salinity) X 100.

Total Bacterial Count (TBC) and Azospirillum Count (Azo.C.) Determinations: After 1.5 and 3 months from start of treatments, rhizospheric soil was microbiologically analyzed for determining the densities of the TBC on nutrient agar medium [34]. *Azospirillum* count was determined on the semi-soil malate medium according to [21] using the most probable number technique (MPN).

Salinity Injuries: After 3 months from start of treatments, the salinity injuries on the treated seedlings were represented as percentage of both the seedlings survival and the injured leaves.

Seedlings survival (%) = (Number of severely injured seedlings / total number of the treated seedlings) x 100.

The seedlings were considered severely injured when more than 50% of their total leaves were shed under salinity.

Injured leaves (%) = (Number of leaves having salinity symptoms / total leaves of seedling) x 100.

Statistical Analysis: The obtained data were statistically analyzed as a factorial experimental design [35] applying the least significant difference (LSD) at 5% for the comparison among the treatment means. Duncan's new multiple range tests and regression analysis was also used.

RESULTS

Growth Characteristics of Seedlings: Generally, most vegetative characteristics of the seedlings were decreased significantly by raising salinity levels, especially at higher levels (14.07EC) compared with that of the control (Table 1). Whereas, the percentages of reduction at the un-inoculated treatment of 14.07EC compared to the control were 20.9%, 26.5%, 50.1%, 46.5%, 51.0%, 56.5% and 63.3% for shoot length, stem diameter, new leaves area/seedling, seedling dry weight, root length, root branch number and root dry weight/seedling, respectively. Applying *Azospirillum* inoculum with normal irrigation (T2) gave significantly higher new leaves area/seedling (87.7cm^2) and root length (33.8cm)

Table 1: Vegetative characteristics of carob seedlings as affected by 4 salinity levels of irrigation water and 2 levels of *Azospirillum lipoferum* inoculation either alone or in combination

Treatments			Shoot length	Stem diameter	New leaves area/seedling	Seedling D.W.[b]	Root length	Root branch	Root D.W./ seedling
No.	Salinity (EC)	Azosp[a]	(cm)	(mm)[c]	(cm^2)	(g)	(cm)	No.	(g)
Salinity levels									
	NW	-------	20.5ab	4.49a	79.6a	3.20a	32.5a	22.3a	0.91a
	4.69	-------	21.9a	4.18b	58.8b	2.76b	31.6a	19.6b	0.74b
	9.38	-------	18.5c	3.93b	46.0c	2.14c	21.1b	18.5b	0.55c
	14.07	-------	17.1c	3.63c	38.9d	1.82d	16.7c	10.8c	0.36d
F-test			***	***	***	***	***	***	***
Azsospirillum inoculation									
	-------	0	19.9a	3.98a	49.9b	2.36b	24.3b	17.8a	0.60b
	-------	1	19.2a	4.13a	61.8a	2.60a	26.5a	17.9a	0.67a
F-test			NS	NS	***	***	*	NS	***
Salinity * *Azospirillum*									
T1	NW	0	21.1a	4.53a	71.5b	3.14a	31.0b	23.0a	0.90a
T2	NW	1	19.8ab	4.50a	87.7a	3.27a	33.8a	21.7ab	0.91a
T3	4.69	0	21.9a	4.37ab	56.4d	2.63c	31.2b	21.5ab	0.70c
T4	4.69	1	22.0a	4.07bc	61.2c	2.90b	32.0ab	18.6c	0.78b
T5	9.38	0	18.7b	3.83c	36.0f	1.99e	20.2c	16.5d	0.48e
T6	9.38	1	18.4b	4.07bc	56.1d	2.30d	22.0c	20.5b	0.61d
T7	14.07	0	16.7c	3.33d	35.7f	1.68f	15.2e	10.0e	0.33g
T8	14.07	1	17.4bc	4.00c	42.1e	1.93e	18.2d	11.5e	0.39f
F-test			**	**	***	***	***	***	***

Values within each column followed by the same letter are not statistically different at 5% level. Measurements were taken on 9-month old seedlings after 3 months from starting treatments. Values are mean of the 2008 and 2009 seasons. D.W.[b], Dry weight; Azosp[a], *Azospirillum lipoferum* inoculation; ([c]), at 5 cm above the soil surface in planting pot; NW, Normal water; NS, Non-significant; *P< 0.05; **P < 0.01; ***P < 0.001; EC, Electrical conductivity

Table 2: Effect of application of 4 salinity levels of irrigation water and 2 levels of *Azospirillum lipoferum* inoculation either alone or in combination on leaf mineral content in carob seedlings

Treatments			Leaf mineral content (%).				
No.	Salinity (EC)	Azosp[a]	Na^+	K^+	Cl^-	N	K^+/Na^+
Salinity levels							
	NW	-------	0.33c	0.74a	0.66c	1.89a	2.27a
	4.69	-------	0.67b	0.54c	0.88b	1.75b	0.81b
	9.38	-------	1.00a	0.52c	1.17a	1.71c	0.51d
	14.07	-------	1.01a	0.67b	1.16a	1.58d	0.67c
F-test			***	***	***	***	***
Azsospirillum inoculation							
	-------	0	0.79a	0.58b	1.01a	1.65b	0.95b
	-------	1	0.72b	0.66a	0.92b	1.82a	1.18a
F-test			***	***	***	***	***
Salinity * *Azospirillum*							
T1	NW	0	0.35f	0.72a	0.63e	1.89a	2.061b
T2	NW	1	0.31g	0.75a	0.68e	1.88a	2.418a
T3	4.69	0	0.69d	0.44e	0.95c	1.62d	0.638ef
T4	4.69	1	0.64e	0.63c	0.82d	1.88a	0.984c
T5	9.38	0	1.07a	0.45e	1.26a	1.63d	0.421g
T6	9.38	1	0.94c	0.57d	1.07b	1.78b	0.606f
T7	14.07	0	1.03b	0.68b	1.21a	1.45e	0.660de
T8	14.07	1	0.97c	0.66b	1.11b	1.71c	0.680d
F-test			***	***	***	***	***

Values within each column followed by the same letter are not statistically different at 5% level. Measurements were taken on 9-month old seedlings after 3 months from starting treatments. Values are mean of the 2008 and 2009 seasons. Azosp[a], *Azospirillum lipoferum* inoculation; NW, Normal water; ***$P < 0.001$

than un-inoculated one (71.5 cm^2 and 31cm, respectively), however, the other vegetative characteristics showed no significant differences. Inoculation of the 4.69EC, 9.38EC and 14.07EC saline water-irrigated seedlings with *Azospirillum* significantly increased new leaves area/seedling, seedling dry weight, root length and root dry weight/seedling compared to the un-inoculated ones under the same level of salinity. Within group data revealed improved responses under all treatment combinations in which the effect of inoculation being more apparent under the higher salinity levels (9.38EC and 14.07EC) than the low level. Whereas, the improving percentages of seedling dry weight were 10.3%, 15.6% and 14.9% for T4, T6 and T8 compared to T3, T5 and T7, respectively.

Leaf Mineral Concentration: The Nå and Cl concentrations were increased significantly in the leaf by raising salinity level, whereas, concentrations of K^+ and N were significantly decreased under the same conditions (Table 2). The accumulations of Na^+ and Cl^- were about 3 and 2 folds, respectively at high salinity level (T7) compared to that in the control (T1). A strong positive correlation between Na^+ and Cl^- accumulation was found (r = 0.960**). While, a negative correlation was found between N concentration and both of Na^+ and Cl^- accumulation (r = -0.714* and -0.811*, respectively). Under normal irrigation, *Azospirillum* inoculum (T2) lead to a significant increase in Na^+ concentration and K/Na ratio in leaf compared to those of un-inoculated ones (T1).

On the other hand, applying *Azospirillum* inoculum in combination with saline water resulted in a significant reduce of the previous salinity effect on Na^+, Cl^-, K^+ and N concentrations in leaf under all tested levels. Since, under the tested salinity levels, the reducing ranges were 0.5-9.5% and 13.6-38.9% for N concentration and 3.8-20.8% and 5.6-38.9% for K^+ concentration, but the raising ranges were 82.6-177.1% and 97.1-205.7% for Na^+ concentration and 30.2-76.2% and 50.8-100.0% for Cl^- concentration in inoculated and non-inoculated seedlings compared with that of the control, respectively.

Also, the K/Na ratio was significantly increased by *Azospirillum* inoculum under the most tested levels of salinity. The highest K/Na ratio (2.46) and the lowest Na concentration (0.31%) were detected in the *Azospirillum*-inoculated seedlings which was significantly lower than

Table 3: Changes in some biochemical contents and total chlorophyll in leaves of carob seedling treated with 4 salinity levels of irrigation water and 2 levels of *Azospirillum lipoferum* inoculation either alone or in combination

Treatments						
No.	Salinity (EC)	Azosp[a]	Proline (mg/g DW[b])	Total Phenols (mg/g DW)	SCC (%)	Total chlorophyll (µg/cm^2)
Salinity levels						
	NW	-------	0.38d	47.65c	4.49c	12.91a
	4.69	-------	0.77c	51.96a	4.36d	11.59b
	9.38	-------	3.95b	48.97b	4.73b	8.18c
	14.07	-------	5.36a	45.79d	5.24a	7.72d
F-test			***	***	***	***
Azsospirillum inoculation						
	-------	0	3.16a	47.43b	4.67a	9.63b
	-------	1	2.07b	49.76a	4.74a	10.57a
F-test			***	***	NS	***
Salinity * *Azospirillum*						
T1	NW	0	0.36f	45.20e	4.45cd	12.97a
T2	NW	1	0.40f	50.09b	4.52c	12.86ab
T3	4.69	0	1.14e	48.01d	4.27d	10.65c
T4	4.69	1	0.40f	55.91a	4.44cd	12.53b
T5	9.38	0	4.89b	48.56cd	4.46cd	07.48f
T6	9.38	1	3.02d	49.39bc	4.98b	08.87d
T7	14.07	0	6.23a	47.94d	5.48a	07.44f
T8	14.07	1	4.49c	43.63f	5.00b	08.00e
F-test			***	***	***	***

Values within each column followed by the same letter are not statistically different at 5% level. Measurements were taken on 9-month old seedlings after 3 months from starting treatments. Values are mean of the 2008 and 2009 seasons. Azosp[a], *Azospirillum lipoferum* inoculation. DW[b], dry weight; NW, Normal water; NS, Non-significant; ***$P < 0.001$

all the other treatments. A significantly negative correlation was found between the K/Na ratio and the accumulated level of both Na^+ and Cl^- (r = -0.898** and -0.878**, respectively).

Biochemical Changes of Leaves

Proline Concentration: The proline concentrations in both non-inoculated and inoculated seedlings increased significantly by raising salinity, especially at higher levels compared with that of the control (T1) (Table 3). The accumulation levels of proline were 3.17, 13.6 and 17.3 fold at salinity levels of 4.69EC, 9.38EC, 14.07EC, respectively. Whereas, the corresponding values under *Azospirillum* inoculum were 1.1, 8.4 and 12.5 fold, respectively. Thus, the *Azospirillum* efficacy on reducing proline accumulation increased significantly by raising salinity levels.

Total Phenols Concentration: There were no significant differences in the total phenol concentrations among the tested salinity levels (Table 3). All the tested salinity levels with or without *Azospirillum* inoculum except for 14.07EC saline treatment significantly increased the concentration of total phenols (47.94-55.91mg/g DW) compared with the control (45.2mg/g DW). Applying *Azospirillum* inoculum gave significantly higher total phenols concentration than uninoculated one. The highest concentration of phenol (55.91 mg/g DW) was found in the combination treatment of saline water at 4.69EC and *Azospirillum* inoculum. Moreover, there was no significant correlation between total phenols and total chlorophylls concentration.

Soluble Carbohydrates Concentration: The SCC in uninoculated seedlings was not significantly different at salinity of 4.69EC and 9.38EC and control (Table 3). Similar results were found in inoculated seedlings or in combination with salinity at 4.69EC compared with the control. At 14.07EC salinity level alone or in combination with *Azospirillum* inoculum resulted in significantly higher SCC percentage (5.48 and 5.00%, respectively) than the other tested treatments (4.27-4.52%), except the combination of 9.38EC salinity and *Azospirillum* inoculum (4.98%).

Table 4: Leaf resistance for water vapor (LRWV), tolerance indices (Ti) and *Azospirillum* efficacy in carob seedling as affected by 4 salinity levels of irrigation water and 2 levels of *Azospirillum lipoferum* inoculation either alone or in combination

Treatments								
No.	Salinity (EC)	Azosp[a]	LRWV[1] sec cm^{-1}	LRWV[2] sec cm^{-1}	Tolerance index (Ti)	*Azospirillum* efficacy (%)	Injured leaves (%)	Survival seedlings (%)
Salinity levels								
	NW	--------	4.39d	3.14d	101.96a	101.96c	00.0d	100
	4.69	--------	11.06c	17.56c	87.92b	105.01b	07.0c	95
	9.38	--------	18.41b	55.16b	68.13c	107.71a	21.6b	85
	14.07	--------	32.22a	96.03a	57.94d	108.43a	46.3a	70
F-test			***	***	***	***	***	-----
Azospirillum inoculation								
	--------	0	13.26b	52.37a	75.07b	100.00b	21.5a	84
	--------	1	19.78a	33.57b	82.86a	111.56a	15.9b	90
F-test			***	***	***	***	**	------
Salinity * *Azospirillum*								
T1	NW	0	04.50e	3.25g	100.0a	-------	00.0d	100
T2	NW	1	04.29e	3.02g	104.0a	103.9c	00.0d	100
T3	4.69	0	10.61d	22.18e	083.9c	-------	14.0c	90
T4	4.69	1	11.51d	12.93f	092.3b	109.9b	00.0d	100
T5	9.38	0	13.25c	64.33c	063.3e	-------	25.7b	80
T6	9.38	1	23.57b	45.98d	073.1d	115.7a	17.5c	90
T7	14.07	0	24.70b	119.72a	053.6f	-------	46.2a	65
T8	14.07	1	39.74a	72.33b	061.5e	114.7a	46.4a	75
F-test			***	***	***	***	***	------

Values within each column followed by the same letter are not statistically different at 5% level. Measurements were taken on 9-month old seedlings after 3 months from starting treatments except LRWV[1] was taken after 1.5 month. Values are mean of the 2008 and 2009 seasons. Azosp[a], *Azospirillum lipoferum* inoculation; NW, Normal water; $^{**}P < 0.01$; $^{***}P < 0.001$. The correlation between LRWV[2] and Ti was -0.951[**]

Total Chlorophyll Concentration: The total chlorophyll concentration of seedling leaves significantly decreased with rising salinity level (Table 3). Under normal irrigation, the seedlings either with or without *Azospirillum* inoculum significantly surpassed the other treated seedlings in the total chlorophyll content. Inoculation of the 4.69EC, 9.38EC, 14.07EC saline water-irrigated seedlings with *Azospirillum* significantly increased total chlorophyll content compared to the uninoculated ones under the same level of salinity.

Physiological Aspects and Azospirillum Efficacy

Leaf Diffusion Resistance to Water Vapour (LRWV): After 1.5 and 3 months from treatments application the LRWV increased progressively by increasing salinity level (Table 4). The range of LRWV increase was 2.4 to 5.5 fold and 6.8 to 36.8 fold at both of the specified two intervals, respectively compared to the control (normal irrigation). There were no significant differences between uninoculated and inoculated seedlings under normal irrigation. At 1.5 month from start of treatments, under salinity levels of 9.38EC, 14.07EC *Azospirillum* inoculum lead to a significant increase in LRWV (23.57 and 39.74 sec cm^{-1}, respectively) compared to those of uninoculated ones (13.25 and 24.70 sec cm^{-1}, respectively). On the contrary, the effects of the combinations of *Azospirillum* inoculum and salinity levels of 4.69EC, 9.38EC, 14.07EC on LRWV were significantly lower than those of the same corresponding levels of salinity alone. Significantly negative correlations were evident between LRWV and all of N concentrations, total chlorophyll and tolerance indices (-0.855**, -0.904** and -0.951**, respectively) (Tables 2, 3 and 4). Otherwise, significantly positive correlations were calculated between LRWV and both Na^+ and Cl^- concentrations (+0.797* and +0.869**, respectively).

Tolerance Index (Ti): Generally, the saline irrigated seedlings either inoculated or non-inoculated with *Azospirillum* significantly reduced the tolerance index compared with that of the control (Table 4). The Ti reduction ranged from 16.1 to 46.4% and from 7.7 to 38.5% for non-inoculated and inoculated seedlings, respectively. However, application of *Azospirillum* inoculum for the seedlings irrigated with any tested salinity level resulted in a significant increase of Ti compared with that of the seedlings under the same solely salinity level. The increase percentages of Ti were 10.8, 15.9 and 15.1 for inoculated seedlings under salinity levels of 4.69EC, 9.38EC and 14.07EC (T4, T6 & T8), respectively.

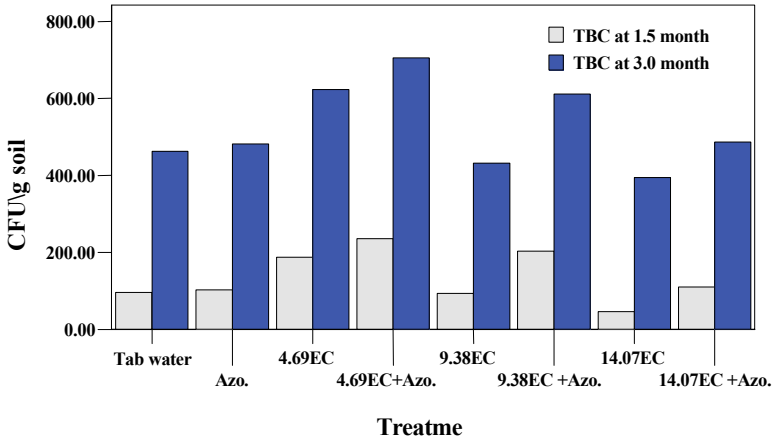

Fig. 1: Changes in the total bacterial count (TBC X 10^6) as affected by 4 salinity levels of irrigation water and 2 levels of *Azospirillum* inoculum (Azo. inoc.) either alone or in combinations

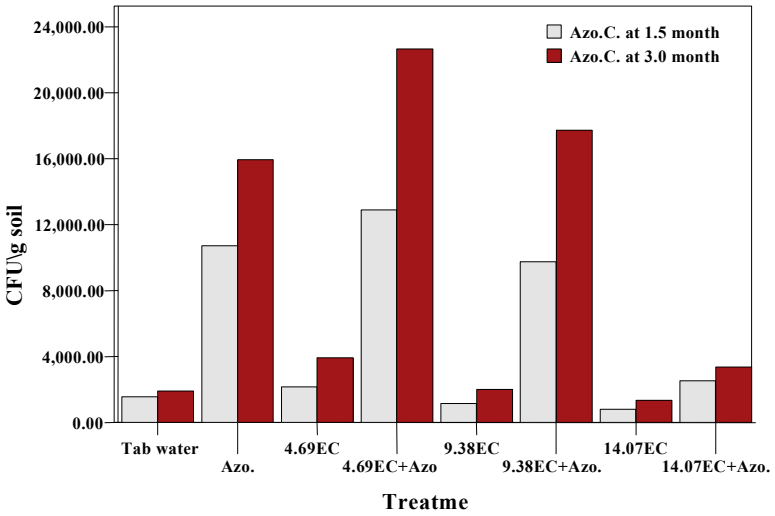

Fig. 2: Changes in *Azospirillum* count (Azo.C.) as affected by 4 salinity levels of irrigation water and 2 levels of *Azospirillum* inoculum (Azo. inoc.) either alone or in combinations

Azospirillum Efficacy: The inoculated seedlings irrigated with saline water at any tested level surpassed significantly in the dry mater accumulation (Azo.E.) compared to the uninoculated corresponding ones (Table 4). Azo.E. value of the treated seedlings increased from 103.9% to 115.7% with increasing the level salinity up to 9.38EC. Such results reflect that the *Azospirillum* inoculation reduced the deleterious salinity effects and had a stimulating effect on synthesis and accumulation of dry mater (biomass production) in the treated seedling.

Salinity Injuries: The percentage of seedlings survival reduced from 90% at the lowest level of salinity (T3) to 65% at the highest level (T7) (Table 4). However, applying *Azospirillum* inoculum under the tested salinity levels maintained higher percentages of seedlings survival than those of the same corresponding levels of salinity alone. On the other hand, the saline irrigated seedlings significantly increased their percentages of injured leaves by about 14.0, 25.7 and 46.2% for T3, T5 and T7, respectively compared with that of the control (T1) (Table 4). Inoculation of the 4.69EC, 9.38EC and 14.07EC saline-irrigated seedlings with *Azospirillum* (T4, T6 & T8 respectively) significantly decreased the injured leaves percentages compared to the uninoculated ones under the same level of salinity (T3, T5 & T7 respectively).

Azospirillum and Total Bacterial Count: After 3 months following commencement of the treatment, TBC was 1.35, 0.93 and 0.64 fold at salinity levels of 4.69EC, 9.38EC and 14.07EC, respectively compared with the control. Whereas, the corresponding values under *Azospirillum*

inoculum were 1.52, 1.32 and 1.06 fold, respectively. At the same time (3 months), *Azospirillum* count was 2.07, 1.05 and 0.70 fold at salinity of 4.69EC, 9.38EC and 14.07EC compared with the control, respectively. Whereas, the corresponding values under *Azospirillum* inoculum were 11.89, 9.31and 1.77 fold, respectively.

Generally, the application of *Azospirillum* inoculum significantly increased the total bacterial count (TBC = 103-482x10^6) and Azospirillum count (Azo.C. = 1.073-1.594x10^4) than those of the control (no inoculation = 96-463x10^6 and 0.157-0.190x10^4, respectively) at both times (Figures 1 and 2). Moreover, inoculation under the salinity levels of 4.69EC, 9.38EC and 14.07EC significantly increased both TBC and Azo.C. compared with those of the same corresponding levels of salinity alone. Such results revealed that both TBC and Azo.C. were progressively reduced by raising salinity level. The highest salinity level (14.07E) gave the lowest values of both TBC and Azo.C. at both counting times. The highest values of both TBC and Azo.C. were expressed by the combination between *Azospirillum* inoculum and 9.38EC salinity.

DISCUSSION

Our results evidenced that the growth characteristics of seedlings (particularly dry weight, new leaf area and root characteristics), concentrations of K^+, N and total chlorophyll, K^+/Na^+ ratio and tolerance index progressively decreased with increased levels of salinity (Tables 1, 2, 3 & 4 and Fig. 1 & 2). These results coincided with those of Please cite the name of the authors (see example paper in the Journal) [36, 37] on maize, [38] on Chinese iris, [39, 14] on *Visia faba*, [40] on guava and [20] on young carob rootstocks.

Salinity stress was also reported to reduce the leaf surface extension rate [41], stunting of plants and considerable decrease in the fresh and dry weights of leaves, stem and roots [42]. Likewise, cite authors names [43] reported a reduction in total plant dry weight and attributed about 80% of the growth reduction at high salinity to the reduction in leaf area expansion and total chlorophyll concentration leading to reduction of light interception and net photosynthetic rate. The remaining 20% of salinity effect on growth is most likely explained by decrease in stomatal conductance.

Reduction of total chlorophyll concentration in carob leaves under salinity in the present study coincided with findings of [44]. This result might be attributed to the decrease in chloroplast number [45] and disorganization of the thylakoid membranes structure of chloroplast [46] in leaves by salt stress.

The decrease in the K^+/Na^+ ratio under salinity (Table 2) might be attributed to an elevation of Na^+ uptake and reduction in K^+ uptake.

On the contrary, the concentrations of proline, Na^+, Cl^- and total phenols, Leaf diffusion resistance to water vapour (LRWV) and leaf injuries (%) significantly increased with increasing the levels of salinity (Tables 2, 3, 4). Such results are in line with those of cite authors names [47] on grapevine, [48] on Cleopatra mandarin, [49] on groundnut and [37] on maize.

In accordance with the finding of [41] on guava, we found a negative relationship between concentrations of Cl^- and Na^+ and concentrations of both K^+ and N in carob leaves. Such results seem to be due to the reduction in N, nitrate and K^+ uptake by the increase in Cl^- [16].

Many plants accumulate proline as a nontoxic and protective osmolyte under saline conditions [50, 51, 52]. Moreover, [53] mentioned that proline accumulation in plants may be a symptom of susceptibility to stress in less salinity-tolerant species and its contribution to osmotic adjustment was apparently negligible as compared with K^+.

The increase in LRWV under *Azospirillum* inculum (Table 4) may possibly be explained by the reduction in root hydraulic conductivity resulting in decreased water flow from roots to shoot [54], which is known to induce Stomatal closure in plants under salinity stress [55]. Salt stress is complex and imposes a water deficit which in turn leads to the formation of reactive oxygen species (O^{-2} and OH), this can seriously disrupt normal metabolism through oxidative damage to lipids and protein [56].

Regarding the inoculation with *Azospirillum* alone or in combination with salinity, our results demonstrate significant increase in some growth parameters (new leaves area/seedling, seedling dry weight, root length and root dry weight/seedling), concentrations of N, total phenols, Soluble carbohydrates and total chlorophyll, K^+/Na^+ ratio and tolerance index compared with the non-inoculated ones (Tables 1, 2, 3 & 4). These results are in agreement with those reported by [57] on pisum, [40] and [14] on *Vicia faba*, [58] on sorghum, [39] on Chinese iris. The initial differences in growth characters between control and stressed seedlings continued by increasing salinity levels but became less pronounced on seedlings inoculated with *Azospirillum*. *Azospirillum*'s growth promotion capacity lies in its ability to produce various phytohormones (axuin and gibberellin) that improve root growth (proliferation, elongation and dry weight),

nitrogen fixation, absorption of water and minerals that eventually result in more plant growth [59, 5, 60, 61]. Also, the involvement of gibberellin produced by *Azospirillum* in promoting growth of inoculated seedlings was suggested [62].

Applying *Azospirillum* inoculum gave significantly higher total phenols concentration than un-inoculated one. Plants are capable of using phenolic compounds as reserve substances for respiratory and other plant metabolism. Furthermore, such a capacity is of great physiological significance in plants that accumulate phenols, the number of which is very considerable [63].

The increase levels of soluble carbohydrates under *Azospirillum* inoculation (Table…) was perhaps due to the necessity of its protective role on chloroplast integrity [46] leading to enhanced photosynthesis under salinity. In this connection, our data with regards to leaf resistance (Table 4) revealed improved conductance under inoculation which may allow better gas exchange and enhancement of photosynthesis.

Otherwise, concentrations of proline, Na+ and Cl-, Leaf diffusion resistance to water vapour (LRWV) and leaf injuries reduced significantly by *Azospirillum* inoculum, alone or in combination with salinity (Tables 2, 3 & 4). The decrease in Na+ concentration in the presence of bio-inoculants might be related to regulation of Na+ uptake and accumulation in the roots thereby delaying its translocation to the shoots [14].

When Azospirillum inoculum takes place under salt stress, plants maintain high concentrations of K+ and low concentrations of Na+ in the cytosol. They do this by regulating the expression and activity of K+ and Na+ transporters and H+ pumps that generate the driving force for transport [64]. Decreasing the accumulation of toxic Cl^- ions in leaves under salinity by *Azospirillum* inoculation probably resulted in adjustment of ABA level which reduces ethylene release and leaf abscission [65].

The values of LRWV declined under *Azospirillum* inoculation at 3 months of treatment commencement (Table 4). This inverse effect of *Azospirillum* inoculation on LRWV allows the plant to decrease traspiratory water loss under prolonged salinity conditions. Such interpretation is in accordance with the finding of cite authors names [66] who found that the members of the genus *Azospirillum* promote plant growth primarily by inducing morphological changes in plant roots leading to enhanced water uptake which cause stomatal opening leading to lowering of LRWV. These results are similar to those found in water-stressed wheat inoculated with *Azospirillum* [67]. They showed better water status and effects on cell wall elasticity and (or) apoplastic water [67].

Our results indicated that improving salinity tolerance of carob seedlings by *Azospirillum* inoculation (Table 4) that reflected by the significant increase in the Ti of inoculated seedlings compared to those of non-inoculated ones under the same level of salinity. Significant positive correlations were found between Ti and all of N concentration, K/Na ratio and total chlorophyll (+0.830*, +0.820* and +0.979**, respectively) (Tables 2, 3 & 4). Otherwise, significant negative correlations were detected between Ti and all of Na^+, Cl^- and proline concentrations, LRWV1 and LRWV2 (-0.903**, -0.962**, -0.968**, -0.788* and -0.951**, respectively). Salt tolerance is usually assessed as the percent of biomass production in saline versus control conditions offer a prolonged period of time for the tolerant species under specified salinity conditions [11]. The highest level of salinity gave rise to the most deleterious effect on Ti, thus, the adverse effect of salinity on plant productivity increases in plants with increasing the level of salinity [68]. It can be noticed that the *Azosprillum* inoculation increased plant tolerance to salinity stress by increasing Ti value. These results are in agreement with those obtained by [68]. Therefore, from the present results it can be noted that determination of Ti along with biomass dry weight represent a reliable criteria for studying the extent of mitigation of adverse salt stress in carob seedling by inoculation with *Azospirillum*.

Count of both total bacteria and *Azospirillum* were increased by *Azospirillum* inoculum under the tested salinity levels (Figs. 1 and 2). These results are in agreement with those obtained by [69] who reported that in soils under oats, the main number of *Azospirillum* in the inoculated combinations were significantly higher than those of the non-inoculated ones. More recent and detailed confirmatory in vitro studies demonstrated that *A. brasilense* Cd can tolerate up to 200 mmol/L NaCl in the medium without appreciable decline in growth. Higher concentrations of salt caused inhibition of bacterial growth. As already known, Azospirillum spp. can accumulate compatible solutes such as glycine betaine, glutamate, proline and trehalose, to allow adaptation to fluctuations in soil salinity/osmolarity [8].

It could be concluded that the non-inoculated seedlings are less salinity tolerant than the inoculated ones. The efficacy of the microorganism in raising the tolerance potential in carob seedlings was due to factors other than proline accumulation such as enhance N and K^+ uptake and reduce Na+ and Cl^- uptake. It was suggest that, under salinity stress, proline accumulation rather than sugars was responsible for salinity tolerance of plants while such mechanism was reversed by the *Azospirillum* inoculation.

CONCLUSION

Based on the results of the present work, it can be concluded that growth of carob seedlings in salinity-affected areas may be remarkably ameliorated by application of *Azospirillum*. This is reflected in improvement of seedling morphological characteristics especially the area of new leaves, dry weight and increased root surface. Likewise, physiological characters such as leaf mineral concentration, LRWV, total chlorophyll and total phenols concentrations were significantly improved by inoculation.

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